

Reduced expression of the genes encoding chloroplast-localized proteins in a cold-resistant *bri1* (*brassinosteroid-insensitive 1*) mutant

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We showed that constitutive activation of the stress-inducible genes led to the endogenous difference to cold tolerance in the *bri1-9* mutant and in the *BRI1-GFP* plants compared to the wild type. In order to get more insight into the balance between growth and stress resistance, we analyzed the cellular localization of the proteins encoded by the genes previously reported as up or down-regulated in the *bri1-9* and *BRI1-GFP* plants. We found that the genes responsible for the chloroplast-localized proteins are markedly downregulated in *bri1-9*, and the proteins encoded by them are involved with chloroplast development, metabolism, and the photosynthetic regulation that is an essential function of chloroplast in the plants cells during active growing periods. These imply that subsets of gene products that are yet uncharacterized modulate the metabolic and signaling processes that are occurring in the chloroplast, leading to the balanced growth and response to abiotic stresses including light stimulus.

Brassinosteroids (BRs) are a plant-specific group of steroid hormones, encompassing more than 60 derivatives, that are synthesized in various monocot and dicot plants. Since their discovery in the early seventies, the role that BRs play in growth regulation, and in particular cell elongation, has been studied intently. Numerous reports have been published highlighting the physiological effects that BRs have on various aspects of plant development in many plant species. Combined biochemical and molecular genetics approaches using BR-biosynthetic and BR-signaling

mutants were used to study the physiological roles of BRs. Biosynthetic pathways and homeostatic control of biosynthesis of BRs have been particularly, as well as its signal transduction mechanisms.¹⁻⁴ BRASSINOSTEROID-INSENSITIVE 1 (BRI1) was originally identified during a screening for the BR-insensitive mutant; BRI1 is a BR receptor⁵ to which BL binds directly.⁶ To date, more than thirty alleles for *bri1* have been reported and mutations have been identified throughout the entire protein.⁷ Depending on the allelic strength, self-fertility can be greatly affected in the individual mutant. Each of the mutant exhibits severe dwarfism, with downward-curved compact rosette leaves, and the leaves of *bri1* mutants are much thicker than those of the wild type plants. Furthermore, the lifespan of the *bri1* mutant plants is extended. Recently, we reported that the *bri1-9* mutant showed a higher tolerance to cold compared to the wild type plant or to the BRI1-overexpressing transgenic plant.⁸

Constitutive Activation of Stress-Inducible Genes in a *bri1* Mutant

The most bioactive BRs are brassinolide (BL), and epi-BL, which is one of the BL-derivatives with a similar physiological function to BL at a higher concentration. Exogenous treatment with these derivatives have been shown to promote the plant tolerance to abiotic stresses such as chilling, heat and high salinity.⁹⁻¹¹ Given this general mode of action, our results that mutant plant defective in BR signaling displayed a more cold-resistant phenotype

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were somewhat unexpected. No study has addressed how BR exerts its anti-stress effects on plants. We therefore, initiated a study on the cellular responses to cold stress in both the *bri1*-mutant and in the *BRII*-overexpressing transgenic plants. The study plants showed opposite growth patterns, facilitating our monitoring of the different physiological responses. Using two plant lines with the wild type plant as a control in this study minimized the ambiguous effects of exogenous treatments with high doses of BL.

We found subsequent morphological changes in the leaves, which developed as dry and fragile. The bleached curing phenotype in our experimental condition (cold stress at 4°C for 24 hours and two-day recovery in normal condition) can be a valid point of reference that can be used to determine the degree of stressed due to cold.⁸ About 45% of the wild type leaves were damaged, but most of the *bri1-9* mutant leaves were not damaged, and the *BRII-GFP* plants exhibited a much higher rate of leaf damage than the wild type. An analysis of the electrolyte leakage before and after the cold stress from these three lines of plants also supported their relative degree of resistance to the cold. These phenomena were not easily explained by the differential expression pattern of several genes in the *CBFs/DREBs* activation pathway that are known to be involved in the cold response.¹² The slightly higher endogenous expression of *CBFs/DREBs*, which might be due to the reduced expression of *HOS1*, a negative regulator of cold response, in *bri1-9* compared to the wild type was not a satisfactory explanation; *BRII-GFP* plants also showed a higher expression of *CBFs/DREBs* compared to the wild type. Moreover, the activation pattern of the *CBFs/DREBs* genes and of the downstream target genes of the *CBFs/DREBs* transcription factors due to cold stress was not distinguishable among *bri1-9*, *BRII-GFP*, and the wild type plants.⁸

This led us to examine the global gene expression and to compare the other genes that show differential expression patterns endogenously in the *bri1-9* and the *BRII-GFP* using microarray analyses. Since the fluctuation pattern of more than 80% of BR-responsive genes upon BL treatment are known to be not so dramatic as the

changing pattern of other plant hormone-regulated genes,¹³ we selected genes that displayed more than a 50% up or 30% downregulated expression compared to wild-type plants after microarray data analyses. There were 238 upregulated genes and 229 downregulated genes in the *bri1-9* mutant. In the *BRII-GFP* plants, 112 and 97 genes were up and downregulated, respectively, (see Appendix S2 to S5 in⁸). We then constructed the functional categorization of these genes and found several noticeable trends. First, 31% of 229 downregulated genes in *bri1-9* and 28% of genes of 112 upregulated genes in *BRII-GFP* plant are involved in metabolic processes, clearly indicating that the metabolic capacities of *bri1-9* are repressed under the normal growth conditions. Secondly, several genes encoding for the ERF (ethylene-responsive factor) subfamily B transcription factors were upregulated in *bri1-9* plants, but were downregulated in *BRII-GFP* plants. The ERF subfamily transcription factors, the DREB subfamily transcription factors to which *CBFs/DREBs* belong, and the RAV transcription factors (*RAV1*, *RAV2*, *RAV1*-like) belong to the stress-inducible AP2 domain-containing group of transcription factors.¹⁴ Third, the *bri1-9* mutants showed higher expression patterns in the stress-inducible genes that might be the target of stress-inducible transcriptional regulators. These stress-inducible genes include several responsive genes for plant hormones; genes involving with late embryogenesis-abundant proteins in seed storage, lipid transfer and dehydration status; and disease resistance genes specific for various pathogens. The results imply that the differential gene expression of the stress-inducible genes in the normal condition led to the endogenous difference to cold tolerance in the *bri1-9* mutant and in the *BRII-GFP* plants compared to the wild type. Even in the absence of pathogen attack or environmental stresses, the *bri1-9* mutant is always alert to other stresses that might be exerted at any time.

Genes Encoding Chloroplast-Localized Proteins in a *bri1* Mutant

In order get more insight into the balance between growth and stress resistance,

here, we analyzed the cellular localization of the proteins encoded by the genes previously reported as up or downregulated in the *bri1-9* and *BRII-GFP* plants by searching a data base (www.ncbi.nlm.nih.gov). Among the 238 upregulated genes in *bri1-9*, three genes are pseudogenes and no localization data is available for 68 of the genes. Similarly, among the 229 downregulated genes in *bri1-9*, one gene is a pseudogene and the proteins encoded by 73 of the genes are not annotated by their localization. For the *BRII-GFP* plants, there is no information about the localization for 29 genes out of the 112 upregulated genes and for 31 genes out of the 97 downregulated genes. We included only the remaining genes in the further analysis of the localization of the gene products; 167 upregulated and 155 downregulated genes in *bri1-9*, 83 upregulated and 66 downregulated genes in *BRII-GFP* plants. We allowed for the redundant counting of the cellular location for these genes, because many proteins encoded by these genes are known to exist in multiple locations, showing ubiquitous expression. As shown in Table 1, the genes encoding for the proteins whose final destinations are in the extracellular region (including the cell wall) and membranes occupied a large portion in each case. One particularly interesting feature that we noticed was that the genes responsible for the chloroplast-localized proteins are markedly downregulated in *bri1-9*. This implies that the functional activities of a group of proteins involved in chloroplast development, or the proteins regulating the metabolic processes occurring in chloroplast may be reduced in *bri1-9*, leading to the extremely retarded growth of the mutant under normal conditions. This assumption led us to look into the functional involvement of the 74 genes whose expression levels were downregulated in *bri1-9*, and into the corresponding gene products that were noted to reside in chloroplast. As we expected, except for 11 hypothetical proteins for which the functions have not been explored, 41 proteins out of the 63 noted in the database (i.e., 65% of the proteins) are involved with chloroplast development, metabolism, and the photosynthetic regulation that is an essential function of chloroplast in the

Table 1. Analysis of the cellular localization of the gene products encoded by the genes differentially regulated in the *brl1-9* mutant and the *BR11-GFP* plants

	No. of gene products by upregulated genes in <i>brl1-9</i>	No. of gene products by downregulated genes in <i>brl1-9</i>	No. of gene products by upregulated genes in <i>BR11-GFP</i>	No. of gene products by downregulated genes in <i>BR11-GFP</i>
Extra cellular including cell wall	41	17	22	15
Plasma membrane	40	18	15	7
Endomembrane	54	42	29	24
Nucleus	28	21	15	19
Cytosol	15	20	7	11
Chloroplast	22	74	19	7
Mitochondria	9	13	5	2
Vacuole	17	6	4	1
Cellular organelles including ER, Goigi, Peroxisome	9	8	6	2

plants cells during active growing periods (Table 2). These results provide additional evidence that the defective growth of the *brl1* mutant resulted from reduced photosynthetic activity due to the lack of chloroplast organization, and in particular photosystem II assembly.

It has long been proposed that BR may have a negative function on light-regulated signaling in plants because of the de-etiolation phenotype that is present in the *det2* mutant (the first identified BR-biosynthetic mutant) under dark conditions.¹⁵ Dark-grown *det2* mutant displayed the increased expression of photosynthetic genes that are normally repressed in the dark. Other BR-biosynthetic and BR-perception mutants such as *cpd*, *dwf4*, *brl1* and *bin2* also generally showed de-etiolation under dark growth and showed abnormal growth in the light.¹⁶⁻¹⁹ However, little is known about the molecular mechanisms that are responsible for this physiological relationship. Recently, the chloroplast protein BPG2 (BRZ-insensitive-pale green 2) was identified from the genetic screening of a mutant that retained pale green cotyledons under light conditions on a media containing brassinazole (BRZ), BR-biosynthetic inhibitor.²⁰ BFG2 functions in BR-mediated chloroplast development through the post-transcriptional accumulation of chloroplast rRNA. We are currently monitoring the effect of the light intensity on the degree of damage caused by cold treatment. We have found that when *Arabidopsis* seedlings are stressed by low temperatures, even the normal intensity growth room lighting conditions

may function as an additional stress (data not shown). Based on our current analysis that is shown in Tables 1 and 2, we propose that, in addition to BFG2, other gene products that are yet uncharacterized modulate the metabolic and signaling processes that are occurring in the chloroplast, leading to the balanced growth and response to abiotic stresses including light stimulus.²¹

Acknowledgements

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Table 2. Analysis of the 74 downregulated genes responsible for the chloroplast-localized proteins in the *bril-9* mutant

Spot number	AGI	Gene description	Average ^a	Localization ^b	Functional involvement ^c
Metabolism					
4684	At4g18810	transcriptional repressor	0.45	CH, Va	transcription repressor activity, regulation of nitrogen utilization
4155	At4g33010	glycine decarboxylase P-protein 1 (AtGLDPI)	0.49	CH, Mito, Ap	glycine cleavage
609	At2g26080	glycine decarboxylase P-protein 2 (AtGLDP2)	0.50	CH, Mito	glycine cleavage
11130	At1g26560	BETA GLUCOSIDASE 40 (BGLU40)	0.54	CH, Ap	carbohydrate metabolic process
8566	At4g36530	hydrolase, alpha/beta fold family protein	0.58	CH	carbohydrate metabolic process
11657	At1g23740	zinc-binding dehydrogenase family protein	0.58	CH, Ap	oxidoreductase activity
7116	At5g13650	elongation factor family protein	0.59	CH	translation elongation factor activity, GTPase activity
10030	At1g17050	Solanesyl diphosphate synthase 2 (SPS2)	0.59	CH	ubiquinone biosynthetic process
5353	At5g42650	allene oxide synthase	0.61	CH, Mito, EM	hydro-lyase activity, response to JA, wounding, fungus
7042	At2g38230	pyridoxine biosynthesis 1.1 (ATPDX1.1)	0.62	CH, Cyto	vitamin B6 biosynthetic process
11140	At4g12830	hydrolase, alpha/beta fold family protein	0.62	CH	carbohydrate metabolic process
8404	At1g32220	hypothetical protein	0.62	CH	coenzyme binding
12523	At5g67030	ABA DEFICIENT 1 (ABA1)	0.64	CH	ABA biosynthesis, response to abiotic stresses
8601	At1g17220	fu-gaeril (FUGI); putative translation initiation factor IF2	0.64	CH	translational initiation
10384	At2g39800	DELTA1-PYRROLINE-5-CARBOXYLATE SYNTHASE 1 (P5CSI)	0.64	CH	response to abiotic stresses, root development
7800	At4g29590	methyltransferase	0.65	CH	metabolic process
7041	At4g33580	BETA CARBONIC ANHYDRASE 5 (BCA5)	0.65	CH	carbon utilization
1129	At5g20070	NUDIX HYDROLASE HOMOLOG 19 (ATNUDX19)	0.66	CH	hydrolase activity, metal ion binding
1469	At4g26530	fructose-bisphosphate aldolase, putative	0.66	CH	glycolysis, metabolic process
9058	At1g68720	TRNA ARGININE ADENOSINE DEAMINASE (TADA)	0.66	CH, PM	hydrolase activity, zinc ion binding, catalytic activity
6957	At3g01180	starch synthase 2 (AtSS2)	0.67	CH	cellulose biosynthetic process
203	At5g48300	ADP GLUCOSE PYROPHOSPHORYLASE 1 (ADGI);	0.67	CH, Ap	photoperiodism, flowering, starch biosynthetic process
677	At5g14660	PEPTIDE DEFORMYLASE 1B (PDF1B)	0.68	CH	translation
6611	At4g08870	arginase	0.68	CH, Mito	polyamine metabolic process
4234	At4g34730	ribosome-binding factor A family protein	0.69	CH	rRNA processing
3598	At3g03780	AtMS2	0.70	CH, Ap, PM	methionine biosynthetic process
10310	At3g04870	ZETA-CAROTENE DESATURASE (ZDS)	0.70	CH	carotene biosynthetic process

^aAverage fold ratio of replicated experiments. ^bCH, chloroplast; Mito, mitochondria; PM, plasma membrane; EM, endomembrane; Va, vacuole; Nu, nucleus; Cyto, cytosol; Ap, Apoplast. ^cSearched from the Entrez Gene in NCBI (http://www.ncbi.nlm.nih.gov/nucore?Db=gene&Cmd=retrieve&dopt=full_report&list_uids).

Table 2. Analysis of the 74 downregulated genes responsible for the chloroplast-localized proteins in the *bril-9* mutant (continued).

Spot number	AGI	Gene description	Average ^a	Localization ^b	Functional involvement ^c
Metabolism (continued)					
7468	At5g04140	GLUTAMATE SYNTHASE I (GLUI)	0.70	CH, Mito, Ap	photorespiration
Chloroplast Development					
7163	At5g15450	CASEIN LYTIC PROTEINASE B3 (CLPB3)	0.55	CH	chloroplast organization
2701	At5g53280	PLASTID DIVISION I (PDVI)	0.63	CH	chloroplast fission
3879	At4g19170	NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 4 (NCED4)	0.65	CH	chloroplast development
4618	At1g03630	PROTOCHLOROPHYLLIDE OXIDOREDUCTASE (POR C)	0.67	CH	chlorophyll biosynthetic process
3885	At3g19720	ACCUMULATION AND REPLICATION OF CHLOROPLAST 5 (ARC5)	0.70	CH	chloroplast fission, GTPase activity
Photosynthetic Activity					
9629	At1g06430	FTSH8	0.57	CH	PSII associated light-harvesting complex II catabolic process
6632	At5g42270	VARIEGATED I (VARI)	0.61	CH	PSII associated light-harvesting complex II catabolic process
4515	At5g58260	hypothetical protein	0.63	CH	NADH dehydrogenase complex (plastoquinone) assembly
12434	At1g50250	FtsH protease I (FTSHI)	0.63	CH	photosystem II repair, PSII associated light-harvesting complex II catabolic process
8549	At1g16720	high chlorophyll fluorescence phenotype 173 (HCF173)	0.64	CH	photosystem II assembly
4127	At1g67840	CHLOROPLAST SENSOR KINASE (CSK)	0.67	CH	photosystem stoichiometry adjustment
7330	At4g28660	PHOTOSYSTEM II REACTION CENTER PSB28 PROTEIN (PSB28)	0.68	CH	photosynthesis
8409	At5g49740	FERRIC REDUCTION OXIDASE 7 (ATFRO7);	0.69	CH	photosynthetic electron transport chain
Protein Modification					
1510	At2g44230	hypothetical protein	0.56	CH	protein myristoylation
2914	At4g31390	ABC1 family protein	0.61	CH	protein phosphorylation
2924	At2g04030	CR88	0.61	CH, Mito, PM	protein folding, response to heat, cold, salt, dehydration, involved in de-etiolation
6269	At1g75460	ATP-dependent protease La (LON) domain-containing protein	0.61	CH	ATP-dependent proteolysis
6204	At3g24190	ABC1 family protein	0.61	CH	protein phosphorylation
1035	At5g57960	GTP-binding family protein	0.63	CH	GTPase activity

^aAverage fold ratio of replicated experiments. ^bCH, chloroplast; Mito, mitochondria; PM, plasma membrane; EM, endomembrane; Va, vacuole; Nu, nucleus; Cyto, cytosol; Ap, Apoplast. ^cSearched from the Entrez Gene in NCBI (http://www.ncbi.nlm.nih.gov/nuccore?Db=gene&Cmd=retrieve&dopt=full_report&list_uids).

Table 2. Analysis of the 74 downregulated genes responsible for the chloroplast-localized proteins in the *bril-9* mutant (continued).

Spot number	AGI	Gene description	Average ^a	Localization ^b	Functional involvement ^c
Protein Modification (continued)					
8147	At2g22360	DNAJ heat shock family protein	0.63	CH	protein folding, response to heat
5088	At1g79600	ABC1 family protein	0.65	CH	protein phosphorylation
10569	At3g19170	PRESEQUENCE PROTEASE 1 (ATPREP1)	0.66	CH, Mito, Ap	protein maturation by peptide bond cleavage
5407	At3g20820	leucine-rich repeat family protein	0.70	CH, Ap	signal transduction, defense response
3769	At4g39960	DNAJ heat shock family protein	0.70	CH	protein folding, response to heat;
Transport					
3744	At3g01550	PHOSPHOENOLPYRUVATE (PEP)/ PHOSPHATE TRANSLOCATOR 2 (PPT2)	0.63	CH	triose phosphate transport
8286	At3g08580	ADP/ATP CARRIER 1 (AAC1)	0.65	CH, Mito, PM, Ap, Va, Nu	purine nucleotide transport;
11670	At5g46800	A BOUT DE SOUFFLE (BOU)	0.66	CH, Mito	ornithine transport
6173	At4g17340	TONOPLAST INTRINSIC PROTEIN 2;2 (TIP2;2);	0.68	CH, Va, PM, EM	transport
Stress-Related					
9653	At2g33380	RESPONSIVE TO DESSICATION 20 (RD20)	0.46	CH, Va, EM	calcium ion binding, response to salt, desiccation, cold
9686	At3g06510	SENSITIVE TO FREEZING 2 (SFR2)	0.68	CH	response to freezing
9939	At3g17800	hypothetical protein	0.68	CH	response to UV-B
8851	At4g34190	STRESS ENHANCED PROTEIN 1 (SEPI)	0.69	CH	response to high light intensity
Development					
848	At4g04020	FIBRILLIN (FIB)	0.45	CH, Nu	structural molecule activity, photoinhibition
6586	At4g22240	plastid-lipid associated protein PAP, putative;	0.64	CH	structural molecule activity
10652	At1g62750	SNOWY COTYLEDON 1 (SCO1)	0.64	CH, Mito, Ap	inflorescence development, negative regulation of seed germination
Unknown					
3417	At5g62140	hypothetical protein	0.44	CH	unknown
6916	At1g16320	hypothetical protein	0.51	CH	unknown
8599	At1g52870	peroxisomal membrane protein-related	0.57	CH	unknown
8337	At1g79510	hypothetical protein	0.58	CH	unknown
5181	At1g50020	tubulin alpha-6 chain like protein	0.58	CH	unknown
547	At2g40400	hypothetical protein	0.62	CH	unknown
6141	At2g35260	hypothetical protein	0.63	CH	unknown
5548	At1g65230	hypothetical protein	0.63	CH	unknown
8178	At3g56360	hypothetical protein	0.65	CH	unknown
4936	At1g12250	thylakoid luminal protein-related	0.69	CH	unknown
4105	At5g02940	hypothetical protein	0.70	CH	unknown

^aAverage fold ratio of replicated experiments. ^bCH, chloroplast; Mito, mitochondria; PM, plasma membrane; EM, endomembrane; Va, vacuole; Nu, nucleus; Cyto, cytosol; Ap, Apoplast. ^cSearched from the Entrez Gene in NCBI (http://www.ncbi.nlm.nih.gov/nucore?Db=gene&Cmd=retrieve&dopt=full_report&list_uids).